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(54) Title: SUBSTRATES FOR NUCLEIC ACID IMMOBILIZATION ONTO SOLID SUPPORTS

(57) **Abstract:** Disclosed is a substrate and process for nucleic acid immobilization on a solid support. A polyanion containing an activated ester is covalently attached to a solid support. In one preferred embodiment, the polyanion is polyacrylic acid.

Substrates for Nucleic Acid Immobilization Onto Solid Supports

Background

1. Field of invention technology

This innovation is concerned with the substrate(s) for nucleic acid(s) immobilization used to produce DNA chip(s). In addition, this innovation is also concerned with the method(s) for the immobilization of nucleic acid onto such substrates and the detection method(s) for the target nucleic acids on that substrate.

2. Related technology

The genome structure of several organisms is revealed according to genome analysis techniques. In parallel with it, the analysis technique for the genome function is developing. In such a situation, DNA chip(s) (DNA microarray) are an attractive technique. A DNA chip is a microarray with many different genes or fragments (DNA fragments) immobilized on the surface of a solid substrate such as a glass slide. A DNA chip (DNA microarray) is useful in the analysis of the expression, mutation, and polymorphism of gene(s).

The method of immobilization of target nucleic acids onto the substrate is the core technology required for the production of DNA chips. There are two primary methods used to produce DNA chips. One method involves the chemical synthesis of the nucleic acids on the tip a linker covalently bound to the substrate, for instance, described in Science, Vol. 251, 767-773 (1991) and Nucleic Acid Research, Vol. 20, 1679-1684 (1992). In this method, DNA is limited to oligonucleotides and a special apparatus is necessary to control the reaction. Thus, it is not a general method.

Another is the method where pre-synthesized DNA or DNA prepared by PCR (Polymerase Chain Reaction) (PCR amplified products) are covalently or non-covalently bound to the substrate, for example, written in Science, Vol. 270, 467-470 (1995) and Nucleic Acid Research, Vol. 22, 5456-5465 (1994).

In the non-covalent immobilization method, the DNA (or RNA) is dissolved in a salt solution such as SSC (sodium chloride/sodium citrate) with or without a denaturing process. The DNA is spotted onto a glass slide coated with basic polycation such as polylysine, polyethylenimine, or silanized with an amine-containing silane compound (3-aminopropyltriethoxysilane for example), and is immobilized by UV-irradiation. All types of DNA (or RNA) should be able to be immobilized by this method.

However, in practice, oligonucleotides and short length DNAs (less than 0.3 Kb) are difficult to immobilize using this method. Even when long length DNA (greater than 0.3 Kb) are attached non-covalently, the processes of washing and hybridization can remove DNA from the substrate, which may cause a reduction in detectability of the target nucleic acid.

Another limitation of the non-covalent immobilization process is increased background signal due to nonspecific binding between residual basic functional groups (cationic) on the substrate surface and the probe nucleic acid (anionic).
It is known that the residual amino groups may be blocked by acetylation (conversion of amines to carboxylic acids using an anhydride such as succinic anhydride). However, the conversion is not always complete.

The development of a substrate, which is applicable for the supersensitive detection of the target nucleic acid, and suitable for the immobilization of many types of nucleic acid, including both short and long length nucleic acids, single stranded nucleic acids, double stranded nucleic acids, DNA, and RNA is desirable.

[p2-p4]

Summary

The primary aims of this invention are: (1) preparation of a novel substrate for nucleic acid immobilization, capable of immobilizing nucleic acids with high efficiency; (2) the substrate immobilized with nucleic acid (i.e. prepared DNA microarrays); (3)

production method for the immobilizing nucleic acids; and (4) detection method for nucleic acid using the substrate immobilized with nucleic acid.

In summary the invention is primarily concerned with the substrate for nucleic acid immobilization; specifically a polyanion based surface for attachment of nucleic acids. In one preferred embodiment, the polyanion is polyacrylic acid, however any polyanion may be used.

Another aspect of the invention involves the immobilization of the nucleic acid to the substrate via a covalent bond. The preferred method involves transforming the carboxylic acid functionalities into activated esters. The activated esters are then reacted with amine modified nucleic acids.

In the primary and secondary embodiments, the substrate is a non-porous surface. A preferred form being glass.

The third aspect of the embodiment is concerned with the substrate immobilized with nucleic acid(s); i.e. the substrate immobilized with nucleic acid, in which nucleic acid is covalently bound to the substrate through the activated ester version of polyanion. In the third embodiment, the polyanion is polyacrylic acid as a preferable form. A preferred activated ester is the pentafluorophenol ester prepared from the polyanion and pentafluorophenol via a carbodiimide coupling.

The fourth embodiment is concerned with the method(s) for nucleic acid immobilization to the substrate; i.e. the method for nucleic acid immobilization to the substrate where the substrate coated with an activated ester derivative of polyanion is contacted with amine-modified nucleic acid. In the fourth embodiment, the polyanion is polyacrylic acid and the substrate is non-porous as the preferable form; glass is the best non-porous substrate.

In the fourth embodiment, the nucleic acid(s) is modified with functional group(s) that has reactivity to the activated ester derivative, in the preferable form the nucleic acid is modified with amino groups.

The fifth embodiment is concerned with the detection method(s) for target nucleic acids in the specimen(s) (or sample(s)) including but not limited to a:

a) Process, in which the substrate for nucleic acid immobilization is the activated ester derivative of the polyanion. The polyanion has been previously covalently attached to the surface of the glass slide.

b) Reaction process between the activated ester derivative mentioned above and the nucleic acid modified with a functional group that has reactivity to the ester derivative.

c) Hybridization process between nucleic acid immobilized on the substrate (target nucleic acid) and complementary nucleic acid (probe nucleic acid).

d) Detection process for hybridized nucleic acids.

The sixth embodiment is concerned with the detection method(s) for nucleic acid in the specimen including but not limited to a:

a) Reaction between the activated ester derivative of the substrate and nucleic acid modified with a functional group that has reactivity to the activated ester derivative.

b) Hybridization process between nucleic acid immobilized on the substrate and complementary nucleic.

c) Detection process for hybridized nucleic acid.

Detailed Description

A polyanion is a polymer that contains two or more negative charges.

The term nucleic acid includes deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the form of an oligonucleotide messenger RNA, anti-sense, plasmid DNA, parts of a plasmid DNA or genetic material derived from a virus (viral DNA) linear DNA, or chromosomal DNA.

A humidostat is any chamber capable of maintaining a fixed humidity range.

In this invention, the substrate(s) for nucleic acid immobilization is not restricted if it is applicable for DNA chips or biosensors. The substrate consists of a solid support

having a non-porous and smooth surface, materials such as glass slides or silica beads are good to use, in the preferable form.

In one embodiment, the substrate is treated with 3-aminopropyltriethoxysilane to provide the surface of the substrate with functional groups capable of reacting with the polyanion (amino groups in this case). Any silane capable of introducing functionality onto the surface of a glass substrate can be used in this innovation. The polyanion can then be covalently attached to the silanated surface of the substrate via a carbodiimide coupling reaction.

Polyanions contain acidic functional groups such as carboxyl groups, phosphate groups, sulfate groups, and so on which have the effect of preventing non-specific binding (electrostatic) with probe nucleic acids. The polyanion is covalently attached to the surface of the substrate; polyacrylic acid, polyglutamic acid, polyaspartic acid, and polyphosphate, are the preferable forms. These polyanions are attached to the silanated surface of the substrate through carbodiimide (or other activated ester chemistry) chemistry.

To immobilize the target nucleic acid onto the polyanion coated substrate it is necessary to activate the acidic functional group of the polyanion (form an activated ester). For example pentafluorophenol and imidazole esters are activated ester derivatives available for the carboxyl group and phosphate group, respectively.

There are no special restrictions placed on the nucleic acid to be immobilized on the substrate. All of the synthesized oligonucleotides, polynucleotides, and their derivatives can be useful. Both single and double stranded forms of nucleic acids (DNA or RNA) are available. The derivatives are not restricted if they have modifications enabling immobilization onto the substrate surface. The derivative(s) modified with amino group, thiol group, phosphate group, and aldehyde group at DNA 5' terminus are examples. In addition, the derivatives modified at 5' terminus with a cross linker or linker as the spacer such as alkylamine are also available. In this innovation, the modification at the 5' terminus is especially effective for the immobilization of short nucleic acids such as oligonucleotide. Any nucleic acid covalently modified to introduce various functional or signaling groups may also be used, Mirus' LabelIT reagents may be used to modify nucleic acids.

Modified nucleic acids mentioned previously are dissolved in the appropriate solution for immobilization, such as 20 mM MOPS (3-morpholino propane sulfate) buffer (pH 7.5) or 50 mM carbonate buffer (pH 9.5) at the concentration of 0.01-2.0 mg/ml, preferably 0.1-1.0 mg/ml. Either with or without a denaturing process, the desired nucleic acid can be immobilized onto the substrate with activated ester functional group by surface treatment, by contact with it. That is, using a micropipette or DNA chip preparation apparatus (DNA arrayer), the constant volume of DNA solution mentioned above was spotted onto the substrate with activated ester functional group by surface treatment as described. It is kept in the humidistat for 30-60 min, and washed with 2% SDS (sodium dodecyl sulfate) and distilled water, respectively. Furthermore, if necessary, the substrate immobilized with nucleic acid is soaked into 0.3 M NaOH at room temperature for 5 min or boiling water for 2 min to denature the nucleic acid, washed with distilled water and absolute ethanol, and dried. By this treatment, the activated ester groups not reacted with nucleic acid in the, are hydrolyzed back to carboxylic acid moieties (i.e. negatively charged). The acid groups prevent nonspecific electrostatic binding between probe nucleic acid and the substrate. In general, the succinic anhydride blocking process necessary for polylysine slides can be omitted. These acid groups also lower the background signal.

The quantity of nucleic acid immobilized on the substrate can be measured by using fluorescently-labeled nucleic acid. Labeled target nucleic acid can be covalently immobilized and residual fluorescent signal detected. The immobilization method in this innovation is capable of supersensitive detection of the expression levels, mutations, polymorphisms, etc, in the gene(s), because this method provides for a high efficiency of immobilization. Furthermore the immobilized nucleic acid will not come off under the hybridization conditions (because the nucleic acid is covalently attached to the substrate).

The general hybridization technique is used for the detection of target nucleic acid covalently attached to the substrate (such as DNA chip). The probe nucleic acid is labeled with a fluorophore (or other signaling group) and is denatured by alkali or heat. The labeled probe nucleic acid is added to the hybridization solution. A 5 μl to 20 μl portion of the hybridization solution is dropped onto the surface of array, overlaid with a cover glass taking care to prevent air bubbles from forming under the cover glass. This is kept

in the humidistat at the appropriate temperature and time. The cover glass is removed, the slide is washed thoroughly, moisture is removed, and finally fluorescent signal is detected by the fluoroscanner (fluorescence reader).

The polyanion coated glass slides specified in this innovation are able to immobilize nucleic acids efficiently. The three-dimensional matrix formed on the surface of the substrate by the covalently attached polyanion allows for the immobilization of increased numbers of nucleic acids.

The substrate and the immobilized nucleic acid in this innovation provide supersensitive detection signal and low background signal detection of nucleic acid. Because the activated ester derivative of polyanion that does not react with the target nucleic acid reverts to polyanion when the activated ester is hydrolyzed, nonspecific binding of negatively charged probe nucleic acid to the substrate is inhibited.

Using this invention, improved sensitivity of target nucleic acid detection can be achieved as a result of: (1) target nucleic acids (including oligonucleotides) are attached to substrate with high efficiency, (2) the blocking process can be omitted after immobilization, (3) the nonspecific binding between the target nucleic acid and the substrate is kept to a minimum, and (4) the 3-dimensional effect of polyanion treatment allowed for high hybridization efficiency.

Examples of Invention

We explain this invention precisely by the following examples. However, this invention is not restricted (limited) to these precise examples.

Example (Illustration) #1

(1) Two kinds of phage DNA fragments (1.0 Kb and 0.3 Kb, respectively, latter is the part of former) shown as Sequence #1 and #2, respectively, in Sequence Listing, were prepared by PCR as the standard DNA.

PCR was performed by using phage DNA as a template and with following two combinations of primers; that of Primer S and Primer A1000 shown as Sequence #3 and #4, respectively, and that of Primer S and Primer A300 as Sequence #5, in Sequence Listing. After that, using Qiaquick PCR purification kit 96 (Qiagen), each amplified

fragment was purified, according to instruction manuals. To elute DNA from column, distilled water was used. DNA was condensed by lyophilization.

Each DNA fragment was dissolved in 20 mM MOPS buffer (pH 7.5), the array was made by GMS 417 arrayer (Genetic MicroSystems) on the glass slide with amino groups on its surface, which is commercially available. The glass slide used was poly-L-lysine coated POLY-PREP-SLIDES (Sigma: abbreviated as PLL coated slide glass) and MAS, amino alkyl silane family, coated slide (Matsunami Glass Ind.: abbreviated as MAS coated slide glass), these slides are widely used for the preparation of DNA chips. The spots were established with the size and intervals of 150 ㎛ and 375 ㎛, respectively. Five spots of same DNA were arrayed.

After DNA spotting, each slide was kept in the incubator controlled at 37 degrees C and 90% humidity for 1 h, and then, UV cross-linked by 60 mJ/cm^2. Slide(s) was washed once with 0.2% SDS and twice with distilled water, removed moisture by centrifugation at low speed (ca.1,000 rpm), followed by air drying. The pre-treated slide mentioned above was washed with distilled water thoroughly, boiled in water bath for 3 min, and exposed in ice cold ethanol to denature DNA. Ethanol was removed by low speed centrifugation. After that, slide was air dried and stored in a desiccator.

A portion of slide(s) was treated with absolute succinic anhydride to block the free amino group(s) on the substrate surface by succinylation. The blocking solution was prepared by the mixing of 1.5 g succinic anhydride resolved in 89.5 ml of N-methyl-2-pyrrolidone with 9 ml of 1 M borate buffer (pH 8.0), the slide was soaked in the blocking solution for 20 min at the room temperature. The treated slide was washed with distilled water thoroughly, boiled in water bath for 3 min, and exposed to ice cold ethanol to denature DNA. Ethanol was removed by low speed centrifugation. After that, slide was air dried and stored in a desiccator.

(2) Commercially available amino alkyl silane glass slides (In situ PCR glass slides, PE Biosystems: abbreviated as AAS slide glass) were soaked into 2.5% glutaraldehyde solution for 1 h, washed with distilled water 3 times, and then air dried. Resulting in a slide coated with aldehyde groups.

The 24 mer oligonucleotide shown as Sequence #6 in Sequence Listing, bound with alkyl amino linker of chain length 6 (PE Biosystems), at 5' terminus (abbreviated as

NH_2-F Oligo), which was prepared on a DNA synthesizer. Then, it was dissolved in 20 mM MOPS buffer (pH7.5) to a final concentration of 10 mM. This synthesized DNA was spotted onto glutaraldehyde treated slides using GMS 417 arrayer in the same manner mentioned in Example Illustration #1-(1). After that, slide glass was washed with 0.2% SDS for 2 min, continuously washed with distilled water. To reduce the Schiff-base, formed by covalent binding between aldehyde group on the substrate and amino group on DNA, into stable amine, the slides were soaked into 0.25% sodium borohydride (Wako Pure Chemical Industries) containing PBS-100% ethanol (volumetric ratio 3:1) for 5 min, and air dried.

(3) Covalent attachment of polyanion to the solid support.

Glass microscope slides were cleaned by ultrasonication in 2 M nitric acid, 2 M sodium hydroxide, pure water, and acetone each for 10 min. The cleaned slides were aminosilinated in 4% 3-aminopropyltriethoxysilane dissolved in 95% ethanol for 2 min, and then incubated at 100□C for 10 min, to aminopropylsilate its surface. Polyacrylic acid was dissolved in dimethylformamide to a final concentration of 5 mg/ml, furthermore, 1-[(3-dimethylamino)propyl-3-ethylcarbodimide hydrochloride (abbreviated as EDC) was added at a final concentration of 4.0 g/100 ml. The aminosilanated slides were soaked in this solution overnight, rinsed with dimethylformamide, methanol, pure water, and acetone. The polyacrylic acid coated slides were dried under vacuum.

(4) Activation of the polyanion coated solid support.

The polyacrylic acid coated slides were converted into activated ester coated slides in the following manner. 1.8 g pentafluorophenol, 2.0 g EDC, and 13 mg dimethylamino-pyridine were dissolved into 50 ml dimethylformamide. The polyacrylic acid coated glass slides prepared in Example Illustration #1-(3) were soaked in this solution for 5 h, rinsed with dimethylformamide and methylene chloride, dried under vacuum, and finally stored in a desiccator.

Example (Illustration) #2

(1) Two sizes of lambda phage DNA fragments (1.0 Kb and 0.3 Kb) were prepared by the same method in Example #1-(1). In this synthesis, DNA fragments with amino groups at the termini were amplified using amine containing Primer S bound with

alkyl amino linker of chain length 6 (PE Biosystems) at the 5' terminus. After purification, each amine-containing DNA fragment was dissolved in 20 mM MOPS buffer (pH 7.5) to a final concentration of 0.5 mg/ml, and then spotted in triplicate onto activated ester coated glass slide activated by the method in Example #1-(4). As the control, the same array was prepared on the PLL coated slide glass.

Activated ester coated glass slides spotted with DNA were kept in the incubator (Tokyo Rikakikai: Eyela model KC-1000) controlled at 37 degrees C and 90% humidity for 30 min, and then, incubated in 0.2% SDS at room temperature to wash out the excess of salt, followed by the wash with distilled water. After that, the slides was soaked in 0.3 N NaOH for 5 min to denature DNA, washed with distilled water thoroughly, dried by centrifugation at about 1,000 rpm, and stored in a desiccator.

(2) Hybridization

Nucleic acid arrays prepared on each glass slide were hybridized with a nucleic acid probe labeled with fluorophore and observed. Each DNA array was pre-hybridized at 42 degrees C for 1 h in the pre-hybridization buffer consisting of 50% formamide, 0.2% SDS, 5x Denhardt' solution, 0.1 mg/ml salmon sperm DNA, and 6x SSC.

The 1.0 Kb lambda phage DNA fragments prepared in Example #1-(1) were labeled with Cy5™ using Label IT® Cy5™ Labeling Kit (Mirus) according to instruction manuals. The labeled nucleic acid was purified by ethanol precipitation. This Cy5™ labeled nucleic acid probe was diluted with pre-hybridization buffer to the final concentrations of 20, 2 and 0.2 ng/ml, heated at 95 degrees C for 2 min, cooled to room temperature. Insoluble matter was removed by centrifugation. About 5 ul of the hybridization solution was dropped onto the nucleic acid array. It was overlaid with a glass coverslip taking care to avoid air bubble formation, and incubated at 42 degrees C for 20 h in the humidistat. The cover glass was removed in the 2x SSC solution at room temperature, washed twice at 65 degrees C for 5 min and at 55 degrees C for 30 min with 0.2% SDS containing 2x SSC. Then, it was washed with 0.05x SSC at room temperature for 10 min, removed moisture by centrifugation at about 1,000 rpm, and air-dried.

After hybridization, the nucleic acid array was read using a GMS 418 array scanner (GMS) with emission wavelength as 635 nm and excitation wavelength as 660

nm. The detected signal strength was analyzed by the image analyzing software ImaGene (Biodiscovery). The result obtained was summarized in Table 1.

Table 1

Probe	DNA fragment immobilized	The substrate for DNA immobilization	
Concentration (ng/ml)	Size (bp)	Activated Ester coated slide glass	PLL coated slide glass
0.2	1000	1484	889
	300	988	856
2	1000	6640	2702
	300	2635	1077
20	1000	27383	14310
	300	7099	3824

As shown in Table 1, at all probe concentration, the signals from each size of immobilized DNA on the activated ester coated glass slides are higher than those on the PLL coated glass slides. That is, in the detection of target PCR amplified nucleic acid fragment, it is shown that DNA chip prepared using activated ester coated glass slides provide higher signal than those prepared on the usual (PLL) coated glass slides.

Example (Illustration) #3

(1) Construction of DNA chip with oligonucleotide-DNA array

Two oligonucleotides of 24 nucleotides in length were prepared on a DNA synthesizer; oligonucleotide shown as Sequence #6 in Sequence Listing, one oligo was modified at the 5' terminus with alkyl amino linker of chain length 6 (PE Biosystems) (abbreviated as NH_2-F Oligo) and another was modified at the 5' terminus to contain an hydroxyl group (abbreviated as OH-F Oligo). Two other oligonucleotides of 24 nucleotides in length were also synthesized; one oligonucleotide having a complementary sequence to the first oligonucleotide shown as Sequence #7, with Cy3^{TM} at 5' terminus (Cy3-R Oligo): and

another oligonucleotide having unrelated sequence shown as Sequence #14, with alkyl amino linker at 5' terminus (NH_2-N Oligo).

OH-F Oligo, NH_2-F Oligo, and NH_2-N Oligo were dissolved in 20 mM MOPS buffer (pH7.5) at a final concentration of 5, 50, and 500 μM, and then arrayed onto activated ester coated glass slide activated by the method in Example #1-(4). At that time, seven spots of each DNA sequence were arrayed. After spotting, the slide was washed and dried as described in Example #2, finally it was treated as oligonucleotide chip. As the control, the same oligonucleotide array was prepared on the glutaraldehyde treated slide as described in Example #1-(2).

(2) Hybridization of oligonucleotide chip

The oligonucleotide chips made on each slide were hybridized with separately prepared Cy3-R Oligos as a probe, and the resulting fluorescent signals were observed. Briefly, about 5 ul of the hybridization solution, consisting of 1 mM Cy3-R Oligo, 0.2% SDS, 5x Denhardts solution, 0.1 mg/ml salmon sperm DNA, and 6x SSC, was added to the oligonucleotide array. It was overlaid with a glass coverslip taking care to avoid air bubble formation and incubated at 42 degrees C for 20 h in the humidistat. The cover glass was removed in the 2x SSC solution at room temperature and washed twice for 10 min in 2x SSC at room temperature. Then, the moisture was removed by low centrifugation at about 1,000 rpm and air-dried. After hybridization, total fluorescence on the oligo-DNA chip was measured using GMS 418 array scanner with emission wavelength as 635 nm and excitation wavelength as 660 nm. The detected signal strength was analyzed by the image analyzing software ImaGene. The result was summarized in Table 2.

Table 2

Concentration of oligonucleotide spotting solution (μM)	Oligo-DNA	Activated Ester coated slide glass	Glutaraldehyde treated slide glass
5	OH-F Oligo	19.8	0.0
	NH_2-F Oligo	5516.7	0.9

	NH_2-N Oligo	0.0	0.0
50	OH-F Oligo	2067.0	0.0
	NH_2-F Oligo	12104.1	0.0
	NH_2-N Oligo	0.0	0.0
500	OH-F Oligo	4385.8	1113.5
	NH_2-F Oligo	14828.7	201.9
	NH_2-N Oligo	0.0	8.5

As shown in Table 2, on activated ester coated slide glass, the spots of oligonucleotide with amino group at the 5' end showed stronger signals than that without amino group. This indicates that the activated ester group on slide glass has reacted with amino group of the oligonucleotide. In all oligonucleotides, the signals from the spots on the activated ester coated glass slides were higher than those on the gluteraldehyde treated glass slide.

Example (Illustration) #4

Three-dimensional effect of polyacrylic acid

The hybridization signals from nucleic acid arrays, on which the fluorescently labeled nucleic acids of different length were spotted, were calculated as signal per nucleic acid length immobilized. A cDNA fragment of human transferrin receptor (abbreviated as TFR) was prepared by the following method. The extraction of mRNA from human cell line K562 (Dainippon Pharmaceutical) and the preparation of cDNA of TFR were performed according to the normal method. With the above cDNA as template, a 4,738 bp PCR product was prepared, using TFR (GeneBank No. X01060) specific primers, Primer S7 and Primer AS7 as Sequence #8 and #9, respectively, in Sequence Listing. The recombinant plasmid pT7TFR was obtained by the ligation of above PCR fragment with pT7Blue vector (Novagen). Fluorescently labeled PCR fragments of different lengths were prepared by the PCR using one primer of the pairs labeled with Rhodamine X (PE Biosystems: abbreviated as ROX) at 5' terminus and pT7TFR as primer and template, respectively; i.e., the PCR was performed using the primer pairs of S7 (Sequence #8) labeled with ROX and AS4 (Sequence #10), AS3 (Sequence #11), AS2

(Sequence #12), or AS1 (Sequence #13). Resulting in 1.0, 0.5, 0.2, and 0.1 kb fragments, respectively, being prepared.

After purification, each DNA fragment was dissolved in 20 mM MOPS buffer (pH 7.5) and aminated using the Amino Label IT® reagent; i.e., the Amino Label IT® reagent was dissolved in dimethyl sulfoxide and added into the purified DNA solution as 1/5 ratio (W/W) and incubated at 37℃ for 1 h. Each aminated DNA was purified by ethanol precipitation method.

The amino Label IT reagent was prepared as described in the patent application WO98/52961, with the displacement of fuluoro-group to amino group.

The aminated TFR fragment was dissolved in 20 mM MOPS buffer (pH 7.5) at a final concentration of 0.5 mg/ml, and then spotted seven points of each sequence by the method of Example #1-(1) onto activated ester coated slide glass activated as Example #1-(4). After spotting, it was washed and dried as described in Example #3. As the control, the same array was prepared on the MAS coated slide glass as Example #1-(1), and treated with or without succinic anhydride blocking of free amino groups after immobilization.

The DNA chips made on each slide glass were hybridized with Cy5 labeled DNA probe, which has a different fluorescent wavelength than ROX, and observed their signals. That is, the 1.0 Kb TFR fragment prepared above was labeled with Cy5™ using Cy5 Label IT® Kit (Mirus) according to manufacturers recommendations.

About 5 ul of the hybridization solution, consisting of 20 ng/ml DNA probe labeled with Cy5, 0.2% SDS, 5x Denhardts solution, 0.1 mg/ml salmon sperm DNA, and 6x SSC, was added onto an area of DNA immobilized on the chip. It was overlaid with the cover glass and incubated at 65 degrees C for 20 h in the humidistat. The cover glass was removed in the 2x SSC solution at room temperature and washed twice at 65 degrees C for 5 min and at 55 degrees C for 30 min with 0.2% SDS containing 2x SSC. Then, it was washed with 0.05x SSC at room temperature for 10 min, removed moisture by low centrifugation at about 1,000 rpm, and air-dried.

After hybridization, the fluorescent signals of ROX labeled at the end of array DNA and Cy5 labeled at hybridization probe on DNA chip were measured using GMS 418 array scanner. The obtained image was analyzed by the image analyzing software

ImaGene and the fluorescent signals were converted to numerical data. The values of the signal strength of Cy5 divided by that of ROX in each DNA size were regarded as the signal strength per DNA. The result was summarized in Table 3. Because of the high background, it was impossible to measure the signals form DNA chip made on MAS coated slide glass without blocking to free amino group.

Table 3

DNA size (bp)	Signal strength per DNA		(A) / (B)
	Activated ester coated slide glass (A)	MAS coated slide glass (B)	
1000	26.77	9.09	2.9
500	9.61	4.17	2.3
200	0.46	0.43	1.1
100	0.30	0.18	1.7

As shown in Table 3, in all DNA fragment length, the signal strength of spots per DNA on the activated ester coated slide glass was higher than that on the MAS coated slide glass. This result indicates that the activated ester coated slide glass allows for more efficient hybridization. The three-dimensional lattice created by the covalently attached polyanion may allow greater access to the attached target nucleic acids.

Example (Illustration) #5

Two types of 1.0 Kb and 0.3 Kb lambda phage DNA fragments with amino group at their ends were prepared by the same method in Example #2-(1). A portion of the DNA was aminated using the Amino Label IT® reagent as described Example #4. Each DNA fragment was dissolved in 20 mM MOPS buffer (pH 7.5) at a final concentration of 0.5 mg/ml, and spotted in seven different places onto activated ester coated slide glass and MAS coated slides.

Nucleic acid arrays made on each slide glass were hybridized with fluorescent Cy3-labeled DNA probes, and the resulting signals were analyzed. That is, a 1.0 Kb lambda phage fragment prepared above was labeled with Cy3 using Label IT® Cy3

Labeling Kit (Mirus) according to the manufacturers recommendations. Using this Cy3 labeled DNA probe, hybridization and washing were performed as the described in Example #4.

Following hybridization, DNA chip fluorescence (i.e. signal intensity) was measured using a GMS 418 array scanner with emission wavelength as 532 nm and excitation wavelength as 570 nm. The obtained images were analyzed and the values of the signal strength were converted to numerical data using the image analyzing software ImaGene. The relative fluorescent strength was calculated by that of spot on MAS coated slide glass as 100. The result obtained was summarized in Table 4.

Table 4

Target DNA		Relative strength of signals	
Size (bp)	Amino modification by Label IT	MAS coated slide glass	Activated ester coated slide glass
1000	-	100	183
1000	+	100	276
300	-	100	945
300	+	100	2492

As shown in Table 4, in all spots of DNA fragments, the signal strength from the activated ester coated slide glass was higher than that from the MAS coated slide glass. Especially the signal for the 300 bp short DNA fragment was much higher than on the MAS slides.

When spotted on the activated ester coated slide glass, target with amine modification produced stronger signals than without amine modification. This demonstrates that the amino modification with Amino Label IT® is effectively provides increased attachment of target to the activated ester coated glass slides.

The foregoing examples are considered as illustrative only of the principles of the invention. Further, since numerous modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and operation shown and described. Therefore, all suitable modifications and equivalents fall within the scope of the invention.

We Claim:

1) A substrate for immobilizing nucleic acid, comprising: a solid support having a surface coated with covalently attachable polyanions for detecting a sample nucleic acid.

2) The substrate of claim 1 wherein the polyanions consist of polyacrylic acid.

3) The substrate of claim 1 wherein the polyanions contain an activated ester.

4) The substrate of claim 3 wherein the activated ester is selected from the group consisting of pentafluorophenol and N-hydroxysuccinimide.

5) The substrate of claim 1 wherein the solid support is non-porous.

6) The substrate of claim 5 wherein the solid support consists of glass.

7) A substrate for detecting nucleic acid, comprising: a solid support having a surface coated with covalently attached polyanions that are attachable to nucleic acid.

8) The substrate of claim 7 wherein the polyanions consist of polyacrylic acid.

9) The substrate of claim 7 wherein the polyanions contain an activated ester.

10) The substrate of claim 7 wherein the solid support is non-porous.

11) The substrate of claim 10 wherein the solid support consists of glass.

12) A process for immobilizing nucleic acid onto a substrate, comprising:

a) coating a solid support with covalently attachable polyanion containing an activated ester;
b) contacting the nucleic acid with the polyanion for detecting.

13) The process of claim 12 wherein the polyanion consists of polyacrylic acid.

14) The process of claim 12 wherein the solid support is non-porous.

15) The process of claim 14 wherein the solid support consists of glass.

16) The process of claim 12 further comprising reacting the nucleic acid with the activated ester.

17) The process of claim 16 wherein the nucleic acid contains an amino group for reacting with the activated ester.

18) The process of claim 12 further comprising a blocking process.

19) A process for detecting a sample nucleic acid in a specimen, comprising:

a) immobilizing the polyanion containing the activated ester of claim 4;
b) reacting the polyanion with an immobilizable nucleic acid containing a functional group that reacts with the ester to immobilize the nucleic acid;
c) hybridizing the immobilized nucleic acid with a sample nucleic acid that is complementary to the immobilized nucleic acid;
d) detecting the hybridized sample nucleic acid.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18573

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12M 1/34; G01N 33/00
US CL :435/287.2; 436/94
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 183, 283.1, 287.1, 287.2; 436/94; 536/23.1, 24.3, 24,33, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YIN et al., Grafting of poly(acrylic acid) onto nonporous glass bead surfaces. Polymers for Advanced Technologies.1997, Vol. 8, Pages 761-766, especially page 761.	1, 2, 5-8, 10, and 11
X — Y	WO 90/05303 (PHARMACIA AB [SE/SE]) 17 May 1990 (17.05.90), see entire document, especially third paragraph of page 15, fourth paragraph of page 16, and second paragraph of page 8.	1-4, 6-9, and 11 ——— 12-16 and 19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 07 AUGUST 2000	Date of mailing of the international search report 28 AUG 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer FRANK LU Telephone No. (703) 308-1235

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	DE 19626750 A1 (F. HERBST) 08 January 1998 (8-01-98), see entire document, especially columns 1, 2, and 5-7.	1, 6, 7, and 11 12-16 and 19
Y	GUO et al. Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. Nucleic Acids Research, 1994, Vol. 22, No. 24, Pages 5456-5465, especially page 5456.	19

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN and WEST
Search terms: DNA, RNA, nucleic acid, oligonucleotide, polyacrylic acid, chip, array, solid support and N-hydroxysuccinimide